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DATA EVALUATION RECORD

[SEQ CHAPTER \h \r 1]**REQUIREMENT:** Not Applicable

TEST MATERIAL (PURITY): *Aedes aegypti* OX5034

SYNONYMS: OX5034

CITATION: Tetracycline-Repressible Transactivator Protein Variant (tTAV - OX5034 OX5034) and Related Genetic Material from OX5034 *Aedes aegypti*: Analysis of No Effect to Threatened or Endangered Species or Critical Habitat. Volume 15, EUP Submission; MRID 50889414, July 16, 2019

SPONSOR: Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX
United Kingdom

AUTHOR: Oxitec Ltd.

TEST SITE: Not applicable

COMPLIANCE: This document is an analysis of the potential impact of OX5034 *Aedes aegypti* on threatened and endangered species or critical habitat. As such, Good Laboratory Practice Standards, 40 CFR Part 160, are not applicable.

This DER does not contain FIFRA CBI.

EXECUTIVE SUMMARY:

The developer of the male-sterile *Aedes aegypti* OX5034 mosquito, Oxitec Ltd., requests an analysis of their finding of no impact to threatened or endangered species or critical habitat (United States Endangered Species Act (ESA) (16 U.S.C. §§ 1531-1544, or Federally designated critical habitat) following release of these mosquitoes for the current Experimental Use Permit (EUP). Oxitec has indicated that the male-sterile Yellow Fever mosquito, *Ae. aegypti* OX5034, will not result in adverse effects to threatened and endangered species based on the following rationale. First, should the OX5034 mosquito be ingested by a listed species, no adverse effect is expected because the tTAV-OX5034 and DsRed2-OX5034 proteins are present at negligible, *de*

minimis, levels in male OX5034 mosquitoes, protein exposure will be transient and minimal due to rapid environmental and gastric degradation, and feeding studies of OX5034 mosquito developmental life stages to predatory fish and invertebrates showed no observable negative effects. Second, OX5034 *Aedes aegypti* is an urban or domestic mosquito closely associated with human habitations. Non-target organisms in these areas are not usually threatened or endangered species. Third, there are no known listed species that are obligate consumers of *Aedes aegypti* mosquitoes or for which *Aedes aegypti* mosquitoes comprise a significant proportion of the diet. Fourth, upon completion of the proposed trial, the population of *Aedes aegypti* is expected to be restored to its pre-field trial population level. Fifth, *Aedes aegypti* is a non-native species in the U.S. and has, therefore, not co-evolved with other organisms in the ecosystem.

PURPOSE OF THE STUDY:

To examine the potential interactions between threatened and endangered species and the male-sterile *Ae. aegypti* OX5034, as well as any influence on critical habitat utilized by threatened and endangered species of all types.

CLASSIFICATION: ACCEPTABLE

I. Description of Product

Strain/Source: OX5034 was developed via standard micro-injection methods (Morris, 1997; Jasinskiene et al., 1998), by injecting a combination of pOX5034 plasmid DNA (containing the tTAV-OX5034 and DsRed2-OX5034 genetic material) and *piggyBac* mRNA as the source of transposase, into *Aedes aegypti* mosquito eggs of an arbovirus free Latin American wild-type strain (originating from Chiapas, Mexico, and held in Oxitec labs since 2006). The transposase mRNA provides a source of *piggyBac* transposase, to allow the rDNA construct to be integrated into the germline of *Aedes aegypti*. The non-autonomous transposon has no endogenous source of transposase in mosquitoes and has had no further translocation. The resulting OX513A line has been maintained in a continuously cycling insect colony for the equivalent of over 27 generation equivalents. Sterile males, homozygous for the two transgenes, are to be released for population suppression; a very low number of homozygous (tTAV-OX5034/ DsRed2-OX5034). When male OX5034 *Aedes aegypti* homozygous for the conditional female-specific self-limiting gene (carrying two copies of the gene) are released into the environment and mate with wild *Aedes aegypti* females, their offspring inherit a single copy (so are hemizygous) of the self-limiting gene. The self-limiting gene kills only female offspring (carrying one copy of the self-limiting gene), which die at early larval stages of development, while hemizygous males will survive to pass the OX5034 genes on further. Hence the OX5034 mosquito can be considered to be a species-specific female larvicide for *Aedes aegypti*.

Function of Product and Mode of Action

The OX513A male-sterile *Ae. aegypti* released into identified receiving environments mate with wild-type *Ae. aegypti* resulting in egg and larval production, however, the presence of the tTAV transgene prohibits the morphogenesis of larvae into adult mosquitoes in the absence of tetracycline. The self-limiting trait is a function of the expression of the tetracycline-repressible transactivator protein variant (tTAV-OX5034) in a female-specific manner. Expression of tTAV-

OX5034 is regulated by tetracycline or one of its derivatives. Tetracyclines bind to tTAV-OX5034 protein, preventing it from activating transcription. Thus, when either tetracycline or one of its analogues is absent from the OX5034 mosquito larval diet, tTAV-OX5034 protein causes lethality in female *Aedes aegypti* carrying at least one copy of the #OX5034 rDNA construct, including the female progeny of matings between OX5034 homozygous males and wild *Aedes aegypti* females.

OX5034 *Aedes aegypti* is a homozygous diploid line of *Aedes aegypti* containing a single integrated copy of the #OX5034 rDNA construct conferring two traits:

- The “conditional female-specific self-limiting” trait, which is conferred through the female-specific expression of a tTAV-OX5034 protein leading to conditional lethality of OX5034, or progeny inheriting the #OX5034 rDNA construct, in the absence of tetracycline or its analogues (conditional lethality is manifested at the early larval stages of development).
- A fluorescent marker, DsRed2-OX5034, to aid in the detection of *Aedes aegypti* containing the #OX5034 rDNA construct. The DsRed2-OX5034 protein belongs to a family of red fluorescent proteins, which are members of a group of non-toxic fluorescent proteins identified in several Anthozoa species. DsRed2-OX5034 is a synthetic sequence variant of the original red fluorescent protein (DsRed) isolated from a coral-like anemone, *Discosoma* sp., that has been modified for faster maturation and lower non-specific aggregation.

To produce OX5034 males for release, larvae are hatched in the absence of tetracyclines. Female larvae die in L2/L3 larval instar stages, while males survive to fully functional adulthood. This means that released OX5034 *Aedes aegypti* will be males that cannot bite humans or other animals and that do not transmit disease.

In OX5034, tTAV-OX5034 protein is only produced in female mosquitoes. This is achieved by linking the tTAV-OX5034 coding sequence to a sex-specific splicing module, which produces different isoforms of tTAV-OX5034 messenger RNA (mRNA) in males and females as a result of alternative splicing. Only the female-specific F2 mRNA isoform can be translated to produce tTAV-OX5034 protein (MRID 50889401).

The male hemizygous OX5034 progeny of matings between released OX5034 homozygous males and wild females can survive in the absence of tetracyclines (though climatic conditions, predation and other factors mean that some will fail to survive to functional adulthood). Any hemizygous OX5034 males that reach adulthood in the wild will be able to pass on the OX5034 rDNA to another generation of progeny, and so on for several generations after the original release of OX5034 homozygous males. To evaluate the persistence of the OX5034 rDNA after cessation of releases, we conducted a trait decline study in a caged population of wild-type *Aedes aegypti*.

A study was conducted to assess whether the male-selecting OX5034 trait would fully select itself out of a wild post-release population at a rate predicted by a stochastic simulation model of natural selection against the introduced trait. Following introduction of the OX5034 male-selecting trait into a caged wild-type population, and subsequent off-tetracycline rearing of future

generations, the male-selecting trait frequency decreased in a manner consistent with model predictions, leading to extinction of the genetic trait after an average of 7 generations post-release, with 10 generations being the longest recorded trait persistence post-release. Full details of this study are provided in (MRID 50889416).

The OX5034 release is intended to reduce, but not eliminate, local *Ae. aegypti* populations. As is similar with chemical (e.g., organophosphate, pyrethroid) or biological (e.g., *Bacillus thuringiensis* var. *israelensis*) mosquito abatement treatments, the decrease in mosquito populations is temporary and populations will rebound from unaffected mosquitoes or gradual movement into the treated area over time. Released male-sterile *Ae. aegypti* applied to a treatment area are anticipated to move less than 200 meters as they are weak fliers and tend to remain primarily in the area of release (LaCroix et al., 2012). Wind storms and occasional animal vectors may transport a small portion of these released mosquitoes outside the immediate treatment areas. Given the short timespan in which these modified insects remain viable (i.e., 2 to 3 days average), they are not expected to spread significantly or establish.

Impacts on Animals from Release of OX5034

The OX5034 *Ae. aegypti* releases will contain 100% male mosquitoes and therefore will not constitute a hazard related to female mosquitoes biting animals or transmitting disease, such as those caused by arboviruses. The OX5034 laboratory colony is evaluated for the presence of several different arboviruses and lots are rejected if the presence of any of these was confirmed.

As noted above, *Ae. aegypti* is predominantly a peridomestic resident and focuses primarily on human hosts when seeking a bloodmeal. The urban nature of this species and its preference for humans as a source of a bloodmeal make interactions with threatened or endangered species far less likely than with many other mosquito species.

Various species of bats, birds, fish, reptiles, amphibians, and insects are known to consume adult or larval mosquitoes with the species of mosquitoes ingested varying by habitat, seasonality and prey preferences. Adult *Ae. aegypti* frequent urban environments predominantly, hence their availability as food for many predatory species will be reduced based upon proximity to human dwellings and the lack of or reduced presence of such predators in the immediate area. Similarly, *Ae. aegypti* preferentially oviposit in clean water associated with various containers (e.g., tires, flower pots, gutters, cisterns) which are commonly associated with human habitation. Hence, consumption of larval *Ae. aegypti* by fish and amphibians is unlikely in such habitat. A study submitted in support of this experimental use permit demonstrated a lack of toxicity or adverse effects to guppies (*Poecilia reticulata*) fed OX5034 larvae (MRID 50698708). American signal crayfish (*Pacifastacus leniusculus*) similarly consumed OX5034 larvae without any evidence of adverse effects (MRID 50698707).

Chiropteran species are considered active generalist predators of insects and it has been anecdotally suggested that insectivorous bats may consume 1000 or more mosquitoes per hour or approximately 12,000 per night. This suggestion stems in part from extrapolations of a study (Griffin et al., 1960) conducted in a sealed environment wherein mosquitoes were the only prey made available to captive bats. The intent of the study was to evaluate echolocation characteristics of *Myotis* spp. in finding *Culex quinquefasciatus*, the southern house mosquito, on

the wing, not to establish the bat's dietary preferences. In areas where larger, more nutritious insect prey are available, bats do not consume large numbers of mosquitoes as they do not constitute significant calories or nutrients relative to the task of preying upon them (Gonsalves et al., 2013; Wetzler and Boyles, 2018). While northern bats (*Myotis septentrionalis*) readily consume mosquitoes in enclosures, evidence suggests that they consume few mosquitoes in an open feeding environment (Boyles et al., 2013).

A study of Big Brown Bats' (*Eptesicus fuscus*) prey preferences, a generalist feeder, indicated a predominance of Coleoptera, Diptera, Ephemeroptera and Lepidoptera species in their diet with Dipteran species predominated by chironomids and very few mosquitoes (Clare et al., 2014). Similarly, a comparison of the diet of eight bat species in southern Illinois concluded that mosquitoes represented a small portion of the overall diet of these insectivorous bats (Feldhamer et al., 2009).

Under certain conditions, such as colder nights where larger insects were less available or when female bats are lactating, Diptera, including mosquitoes and crane flies, may constitute a larger portion of the diet of the southeastern brown bat, *Myotis austroriparius*, in Florida (Zinn and Humphrey, 1983). These Dipterans constituted as much as 75% of biomass sampled by bats on cooler nights, however, the diversity of the diet of this insectivorous bat increased considerably during warmer temperatures (*i.e.*, most spring and summer nights). In a recent Wisconsin study, little brown bats (*Myotis lucifugus*) and big brown bats (*Eptesicus fuscus*) were found to include mosquitoes (9 species identified) in their diet at 72% and 33% of samples, respectively, at all sites sampled (Wray et al., 2018). In contrast, Whitaker and Lawhead (1992) found mosquitoes in 17% of fecal samples of *M. lucifugus* which constituted 1.8% (volumetrically) of their insect-based diet. Given the taxonomic, temporal and geographical breadth of the studies referenced above, it can be concluded that for different insectivorous bat species, mosquitoes may constitute less or more of their overall dietary intake of insects depending in part on seasonality, bat species and availability of diverse prey.

Numerous insectivorous avian species include mosquitoes within their diet, however, none are known to include a significant biomass of mosquitoes as a regular part of their consumed prey. Purple Martins (*Progne subis*) have been considered as actively seeking mosquitoes among other flying insects when feeding. A study in Oklahoma evaluating prey captured by Purple Martins at various altitudes failed to detect mosquitoes as part of prey fed to young birds (Helms et al., 2016). Other reports suggest that mosquitoes typically do not make up more than 3% of the Purple Martin diet (Miller, 2006).

In a seven-year study conducted in Edinboro, PA, mosquitoes were not detected in 500 Purple Martin diet samples collected and analyzed (PMCA, 2006). Based upon the feeding habits of Purple Martins, it was considered as unlikely that the birds and freshwater mosquitoes would encounter each other routinely.

In contrast, Western Bluebirds, *Sialia mexicana*, consumed *Aedes* (species not identified) as the most common arthropod prey among 66 species identified in fecal samples (Jedlicka et al., 2017) from California vineyards. Samples from adults and nestlings indicated 51% and 49%, respectively, contained *Aedes* as prey.

Reptiles and amphibians are known to predate on mosquitoes, both adult and larval stages. While neither group has evolved to specifically target mosquitoes as a major portion of their diet, in some instances, mosquitoes can constitute a significant source of prey. The Tiger Salamander, *Ambystoma tigrinum*, was found to readily consume mosquito (*Culicidae*) larvae based on 26% of stomach samples containing remnants of larvae (Brodman and Dorton, 2006). Interestingly, salamander length was negatively correlated with the number of mosquito larvae consumed but positively correlated with consumption of larger prey (e.g., tadpoles, beetles). Mosquito larvae were determined to be the third most commonly consumed prey in this pond study. Eastern Spotted Newts, *Notophthalmus viridescens*, were also found to consume mosquito larvae among other insect prey in aquatic situations (Matheson and Hinman, 1929).

In addition to direct consumption, mosquito larvae and amphibian larvae (i.e., tadpoles) may directly compete for resources and impact growth of each other, as well as survival (Mokany and Shine, 2003). While most tadpoles are herbivorous, some species will consume mosquito larvae and other insects in certain situations. It has been suggested that some mosquitoes will preferentially oviposit in waters with few or no tadpoles present.

Exposure of listed plant species is expected to be minimal. *Aedes aegypti* are primarily found in urban areas and have minimal interaction with terrestrial plant species in natural ecosystems or agricultural crops, beyond adults nectar feeding on flowers for carbohydrates, and specific species (e.g., the Bromeliad family) that provide an oviposition substrate in the parts which retain water, in the urban environment. In addition, *Aedes aegypti* do not pollinate plants. The tTAV-OX5034 and DsRed2-OX5034 proteins are expressed in OX5034 tissues within the confines of its chitinous exoskeleton and, therefore, are unavailable to plants. Plant leaves generally have an external waxy cuticle, which repels water and facilitates the physical removal of contaminants. It is highly unlikely that the rDNA construct could be transferred to other species that may be involved in pollination of plant. As a result of these combined factors, TAV-OX5034 and DsRed2-OX5034 proteins are unlikely to result in adverse effects on listed plant species.

BPPD Comments:

Based upon bioinformatic analyses, neither DsRed2-OX5034 or tTAV-OX5034 are known to share significant sequence homology with known toxins (MRID 50889420). The tTAV-OX5034 protein does not share significant homology with any known or putative allergens, however the Agency has not made a determination regarding the allergenicity of DsRed2. Both of these proteins are predicted as susceptible to several proteases found in the human gastric system (i.e. pepsin, trypsin, chymotrypsin) based upon bioinformatics analysis (MRID 50889420), thus proteins are expected to be broken down following ingestion. Based upon bioinformatics analyses, both DsRed2-OX5034 and tTAV-OX5034 are also predicted as susceptible to two environmental proteases (i.e. proteinase K and subtilisin A) and are thus expected to degrade under field conditions. While several variants of DsRed can sometimes exhibit toxic effects within living cells, oral consumption and subsequent digestion would result protein degradation, thus uptake of the intact protein into cells following ingestion is unlikely. Because biting females will not be released, wildlife will not serve as bloodmeals for mosquitoes carrying tTAV-

OX5034 and DsRed2-OX5034 proteins, thus excluding this as an exposure pathway to these proteins. These proteins are not expected to persist in the environment nor in the tissues of any animals ingesting mosquitoes containing these proteins.

Aedes aegypti is known to frequent households and associated habitat in close proximity to buildings inhabited by humans, thus limiting exposure for listed plants and wildlife. Furthermore, biting females will not be released thus there would be no exposure to tTAV-OX5034 and DsRed2-OX5034 proteins via this route. Lastly, the proteins are predicted a susceptible to gastric and environmental proteases and will thus be subjected to digestion once ingested and degrade once in the environment. The presence of OX5034 male mosquitoes in the environment is not anticipated to present a risk to threatened or endangered species nor negatively impact the habitat of such species. The submitted analysis is appropriate considering the minimal exposure of threatened and endangered species and their critical habitat from release to OX513A male mosquitoes for the current EUP.

CONCLUSION: ACCEPTABLE

REFERENCES:

- Boyles, J.G., Catherine L. Sole, C.L., Cryan, P.M., and G.F. McCracken (2013) On Estimating the Economic Value of Insectivorous Bats: Prospects and Priorities for Biologists, *In*, R.A. Adams and S.C. Pedersen (eds.), *Bat Evolution, Ecology, and Conservation*, DOI 10.1007/978-1-4614-7397-8_24, © Springer Science+Business Media New York
- Brodman, R. and Dorton, R. (2006) The Effectiveness of Pond-Breeding Salamanders as Agents of Larval Mosquito Control, *Journal of Freshwater Ecology*, 21:3, 467-474.
DOI: 10.1080/02705060.2006.9665024
- Canyon D.V. and Hii, J.L. (1997) The gecko: an environmentally friendly biological agent for mosquito control. *Med Vet Entomol.* Oct;11(4):319-23
- Clare, E.L., Symondson, W.O.C., and Fenton, M.B. (2014) An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). *Molecular Ecology* (2014) 23, 3633–3647
- Feldhamer GW, Carter TC, Whitaker JO. 2009. Prey consumed by eight species of insectivorous bats from southern Illinois. *The American Midland Naturalist*, 162(1):43-51.
- Gill, G., Ptashne M. (1988) Negative effect of the transcriptional activator GAL4. *Nature* 334, 721-724. August 25, 1988.
- Gonsalves L., Bicknell B., Law, B., Web, C., Monamy V. (2013). Mosquito Consumption by Insectivorous Bats: Does Size Matter? *PLoS ONE* 8(10): e77183.
doi:10.1371/journal.pone.0077183
- Goodman RE. (2017) Updated bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases. Unpublished report prepared by Food Allergy Research and Resource Program. Food Science and Technology. University of Nebraska. Study Number: REG Oxitec OX513A_2. January 8, 2017. 132 p. EPA MRID 50326403.
- Griffin, D.R., Webster, F.A., and Michael, C.R. (1960) Echolocation of flying insects by bats. *Animal Behaviour* 8(3-4):141-154.
- Helms J.A., Godfrey A.P., Ames T., Bridge E.S. (2016). Are invasive fire ants kept in check by native aerial insectivores? *Biol Lett.* 2016 May;12(5). pii: 20160059. doi: 10.1098/rsbl.2016.0059.
- Jasinskiene N1, Coates CJ, Benedict MQ, Cornel AJ, Rafferty CS, James AA, Collins FH. 1998. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. *Proc. Natl. Acad. Sci. U.S.A* 95(7):3743--3747.

Jedlicka, J.A., Vo, A.-T. E., and Almeida, R.P.P. (2017) Molecular scatology and high-throughput sequencing reveal predominately herbivorous insects in the diets of adult and nestling Western Bluebirds (*Sialia mexicana*) in California vineyards. *The Auk* 134: 116-127.

Lacroix, R., McKemey, A.R., Raduan, N., Wee, L.K., Ming, W.H., Ney, T.G., Siti Rahidah A.A., Salman, S., Selvi Subramaniam, Oreenaiza Nordin, Norhaida Hanum A.T., Chandru Angamuthu, Suria Marlina Mansor, Rosemary S. Lees, Neil Naish, Sarah Scaife, Pam Gray, Genevieve Labbe', Camilla Beech, Derric Nimmo, Luke Alphey, Seshadri S. Vasan, Lee Han Lim, Nazni Wasi A., Shahnaz Murad (2012) Open Field Release of Genetically Engineered Sterile Male *Aedes aegypti* in Malaysia. *PLoS One* 7(8): e42771. doi:10.1371/journal.pone.0042771

Lin H., McGrath J., Wang P., Lee T. (2007) Cellular toxicity induced by SRF-mediated transcriptional squelching. *Toxicol Sci* 96: 83-91.

Matheson, R. and E.H. Hinman. (1929). The vermilion spotted newt (*Diemictylus viridescens*) as an agent in mosquito control. *Am. J. Hygiene* 9: 188-191.

Miller J. (2006) Purple Martin Mania. *Missouri Conservationist Magazine*: March 2006 Issue: <https://mdc.mo.gov/conmag/2006/03/purple-martin-mania>

Mokany, A. and R. Shine (2003) Competition between tadpoles and mosquito larvae. *Oecologia* 135:615-620

Morris AC. (1997). *Microinjection of Mosquito Embryos*. Chapman and Hall, London.

Nordin O., Donald W., Ming W., Ney T., Mohamed K., Halim N., Winskill P., Hadi A., Muhammad Z., Lacroix R. et al., (2013) Oral Ingestion of Transgenic RIDL *Ae. aegypti* Larvae Has No Negative Effect on Two Predator *Toxorhynchites* Species. *PLoS ONE* 8: e58805 doi:10.1371/journal.pone.0058805. (Volume 8 of the EUP application).

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., Coleman, P.G., White-Cooper, H. and Alphey, L. (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5:11 doi:10.1186/1741-7007-5-11 [HYPERLINK "https://bmcbiol.biomedcentral.com/articles/10.1186/1741-7007-5-11"]

Purple Martin Conservation Association (PMCA) (2006) The Purple Martin Conservation Association in cooperation with Edinboro University of Pennsylvania. <http://www.purplemartin.org>

USDA-FS, United States Department of Agriculture, Forest Service and Department of Interior, Bureau of Land Management (2009) Species Fact Sheet – Purple Martin, Updated May 2009, by Rob Huff, Oregon State Office, BLM [HYPERLINK "https://studylib.net/doc/7750091/species-fact-sheet---usda-forest-service"] [accessed 11-20-18]

Wetzler G.C and Boyles J.G. (2018). The energetics of mosquito feeding by insectivorous bats. *Canadian Journal of Zoology*. 96: 373–377.

Whitaker J.O and Lawhead B. (1992). Foods of *Myotis lucifugus* in a maternity colony in central Alaska. *J. Mammal* 73:646–648.

Wray A.K, Jusino MA, Banik M.T, Palmer JM, Kaarakka H, Whit J.P. (2018). Incidence and taxonomic richness of mosquitoes in the diets of little brown and big brown bats. *Journal of Mammalogy* 99 (3), 668-674.